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SITE LOGIC Report

QuantArray®-Petro Study

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Report Date: 05/18/2020

Project: Williams AFB ST012 EBR, 9101110001.5310.02
Comments:

The QuantArray®-Petro Approach

Comprehensive evaluation of biodegradation potential at petroleum impacted sites is inherently problematic due to two factors:

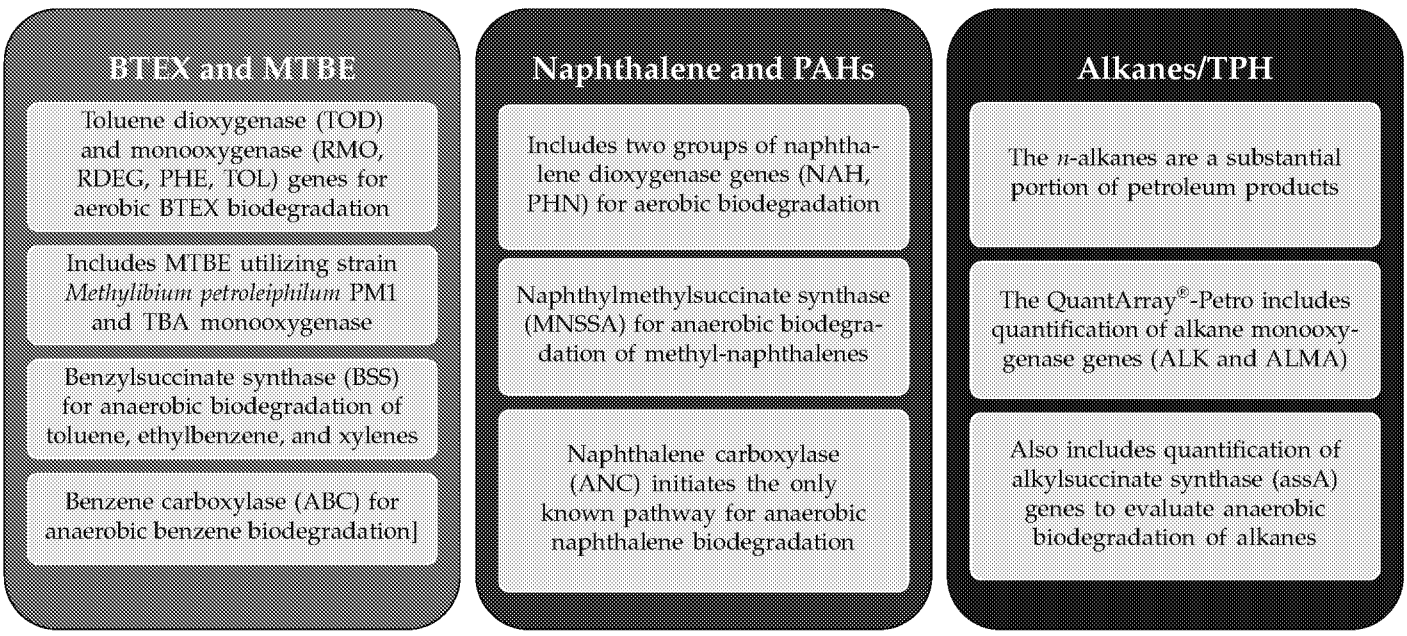
(1) Petroleum products are complex mixtures of hundreds of aliphatic, aromatic, cyclic, and heterocyclic compounds.

(2) Even for common classes of contaminants like benzene, toluene, ethylbenzene, and xylenes (BTEX), biodegradation can proceed by a multitude of pathways.

The QuantArray®-Petro has been designed to address both of these issues by providing the simultaneous quantification of the specific functional genes responsible for both aerobic and anaerobic biodegradation of BTEX, PAHs, and a variety of short and long chain alkanes.

Thus, when combined with chemical and geochemical groundwater monitoring programs, the QuantArray®-Petro allows site managers to simultaneously yet economically evaluate the potential for biodegradation of a spectrum of petroleum hydrocarbons through a multitude of aerobic and anaerobic pathways to give a much clearer and comprehensive view of contaminant biodegradation.

The QuantArray®-Petro is used to quantify specific microorganisms and functional genes to evaluate aerobic and anaerobic biodegradation of the following classes of compounds present in petroleum products:



How do QuantArrays® work?

The QuantArray®-Petro in many respects is a hybrid technology combining the highly parallel detection of microarrays with the accurate and precise quantification provided by qPCR into a single platform. The key to highly parallel qPCR reactions is the nanoliter fluidics platform for low volume, solution phase qPCR reactions.

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How are QuantArray® results reported?

One of the primary advantages of the QuantArray®-Petro is the simultaneous quantification of a broad spectrum of different microorganisms and key functional genes involved in a variety of pathways for hydrocarbon biodegradation. However, highly parallel quantification combined with various metabolic and cometabolic capabilities of different target organisms can complicate data presentation. Therefore, in addition to Summary Tables, QuantArray®-Petro results will be presented as Microbial Population Summary and Comparison Figures to aid in the data interpretation and subsequent evaluation of site management activities.

Types of Tables and Figures:

Microbial Population Summary	Figure presenting the concentrations of QuantArray®-Petro target gene concentrations (e.g. toluene dioxygenase) relative to typically observed values.
Summary Tables	Tables of target population concentrations grouped by biodegradation pathway and contaminant type.
Comparison Figures	Depending on the project, sample results can be presented to compare changes over time or examine differences in microbial populations along a transect of the dissolved plume.

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Results

Table 1: Summary of the QuantArray®-Petro results obtained for samples UWBZ26-QA-050520, UWBZ27-QA-050520, LSZ38-QA-050520, and LSZ39-QA-050520.

Sample Name Sample Date	UWBZ26-QA-050520 05/05/2020	UWBZ27-QA-050520 05/05/2020	LSZ38-QA-050520 05/05/2020	LSZ39-QA-050520 05/05/2020
Aerobic BTEX and MTBE	cells/mL	cells/mL	cells/mL	cells/mL
Toluene/Benzene Dioxygenase (TOD)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Phenol Hydroxylase (PHE)	**2.36E+04**	**9.27E+04**	**4.22E+04**	**4.06E+04**
Toluene 2 Monooxygenase/Phenol Hydroxylase (RDEG)	**1.56E+04**	**6.66E+04**	**2.93E+04**	**2.37E+04**
Toluene Ring Hydroxylating Monooxygenases (RMO)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Xylene/Toluene Monooxygenase (TOL)	**1.68E+02**	**9.04E+02**	**2.52E+02**	**1.67E+03**
Ethylbenzene/Isopropylbenzene Dioxygenase (EDO)	**2.40E+00 (J)**	**9.40E+00**	**8.80E+01**	**7.17E+01**
Biphenyl/Isopropylbenzene Dioxygenase (BPH4)	<2.00E+01	**2.29E+01**	<2.27E+01	<2.27E+01
Methylibium petroleiphilum PM1 (PM1)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
TBA Monooxygenase (TBA)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Aerobic PAHs and Alkanes				
Naphthalene Dioxygenase (NAH)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Naphthalene-inducible Dioxygenase (NidA)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Phenanthrene Dioxygenase (PHN)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Alkane Monooxygenase (ALK)	**7.00E+00 (J)**	<4.60E+00	<2.27E+01	**1.89E+01 (J)**
Alkane Monooxygenase (ALMA)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Anaerobic BTEX				
Benzoyl Coenzyme A Reductase (BCR)	**9.45E+02**	**3.19E+02**	**3.62E+01**	**9.49E+02**
Benzylsuccinate Synthase (BSS)	**1.04E+04**	**9.65E+02**	**8.50E+02**	**9.00E+03**
Benzene Carboxylase (ABC)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Anaerobic PAHs and Alkanes				
Naphthylmethylsuccinate Synthase (MNSSA)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Naphthalene Carboxylase (ANC)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Alkylsuccinate Synthase (ASSA)	**1.20E+02**	**6.10E+00**	<2.27E+01	**1.14E+01 (J)**
Other				
Total Eubacteria (EBAC)	**3.47E+07**	**1.34E+07**	**4.82E+06**	**2.22E+07**
Sulfate Reducing Bacteria (APS)	**3.59E+06**	**5.18E+06**	**2.53E+06**	**2.65E+06**

Legend:
NA = Not Analyzed
NS = Not Sampled
J = Estimated Gene Copies Below PQL but Above LQL
I = Inhibited
< = Result Not Detected

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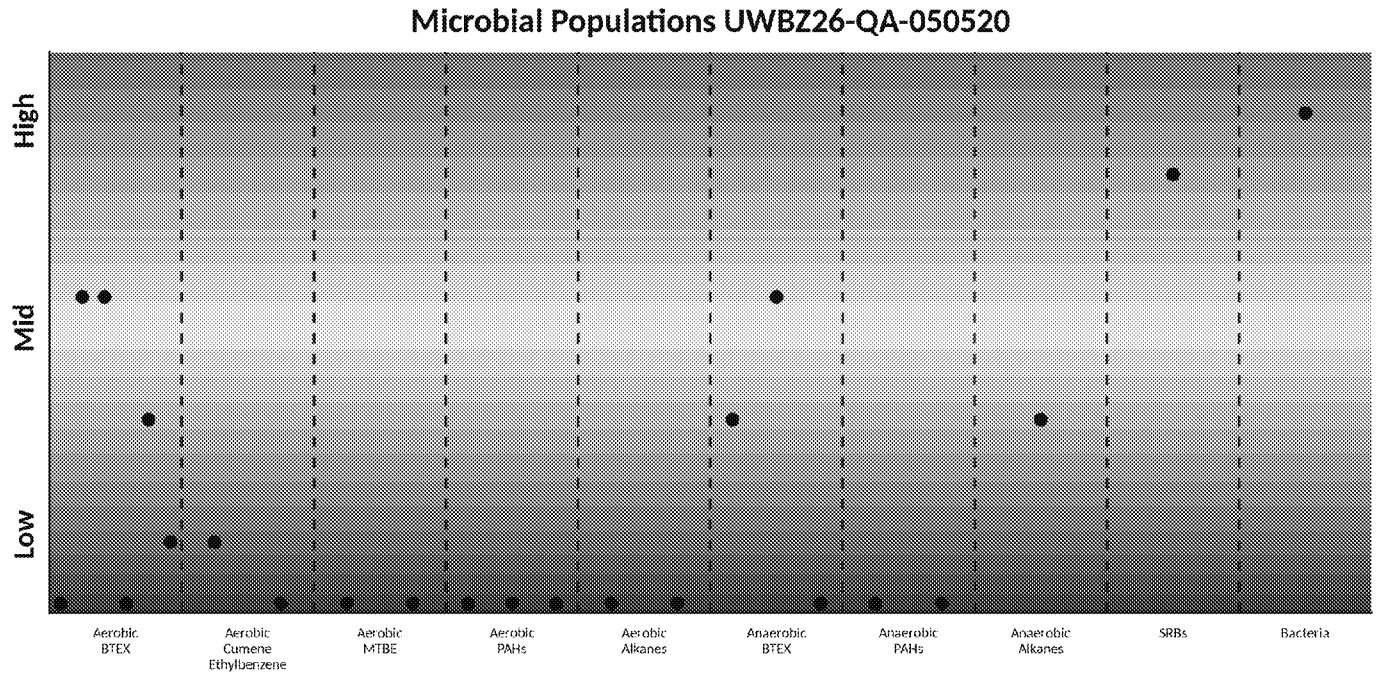

Figure 1: Microbial population summary to aid in evaluating potential pathways and biodegradation of specific contaminants.

Aerobic		Anaerobic	
BTEX	TOD, PHE, RDEG, RMO, TOL, EDO	BTEX	BCR, BSS, ABC
Cumene, Ethylbenzene	EDO, BPH4	Naphthalene/Methylnaphthalene	MNSSA, ANC
MTBE/TBA	PM1, TBA	Alkanes	assA
Naphthalene	NAH, NidA		
Phenanthrene	PHN		
Alkanes	ALK, ALMA		

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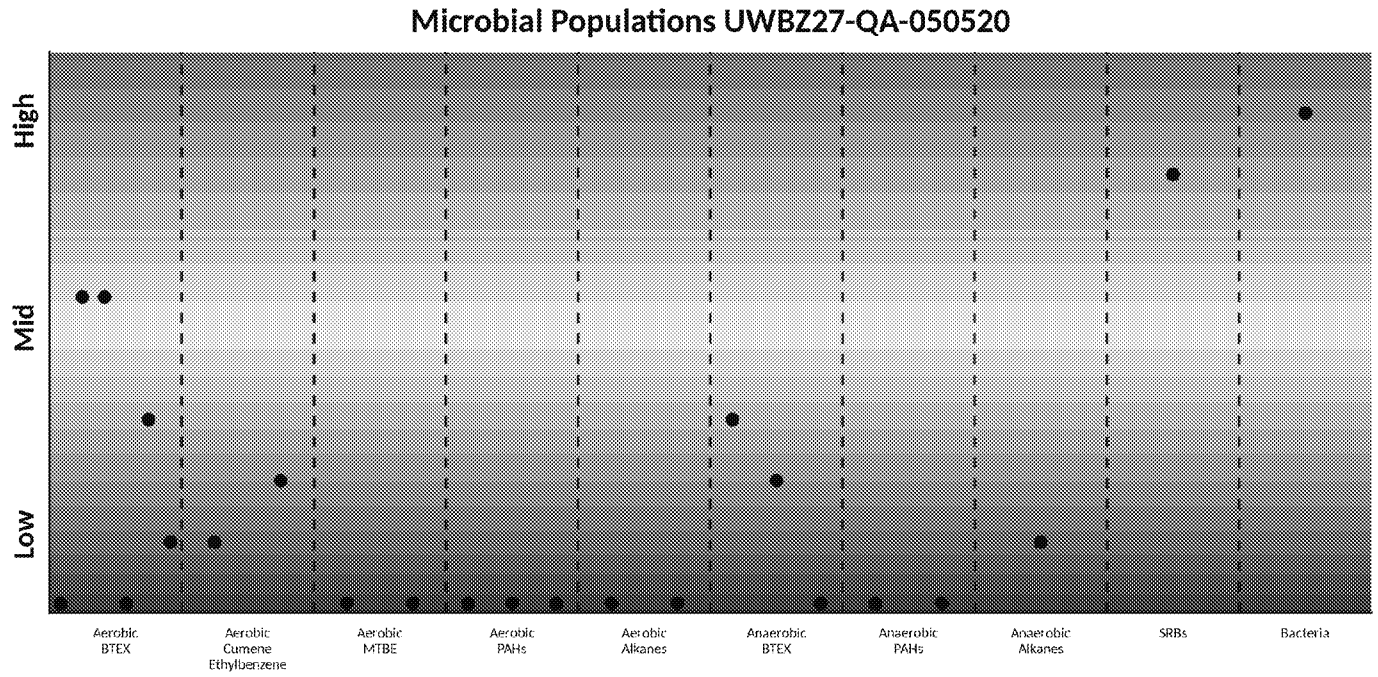

Figure 2: Microbial population summary to aid in evaluating potential pathways and biodegradation of specific contaminants.

Aerobic		Anaerobic	
BTEX	TOD, PHE, RDEG, RMO, TOL, EDO	BTEX	BCR, BSS, ABC
Cumene, Ethylbenzene	EDO, BPH4	Naphthalene/Methylnaphthalene	MNSSA, ANC
MTBE/TBA	PM1, TBA	Alkanes	assA
Naphthalene	NAH, NidA		
Phenanthrene	PHN		
Alkanes	ALK, ALMA		

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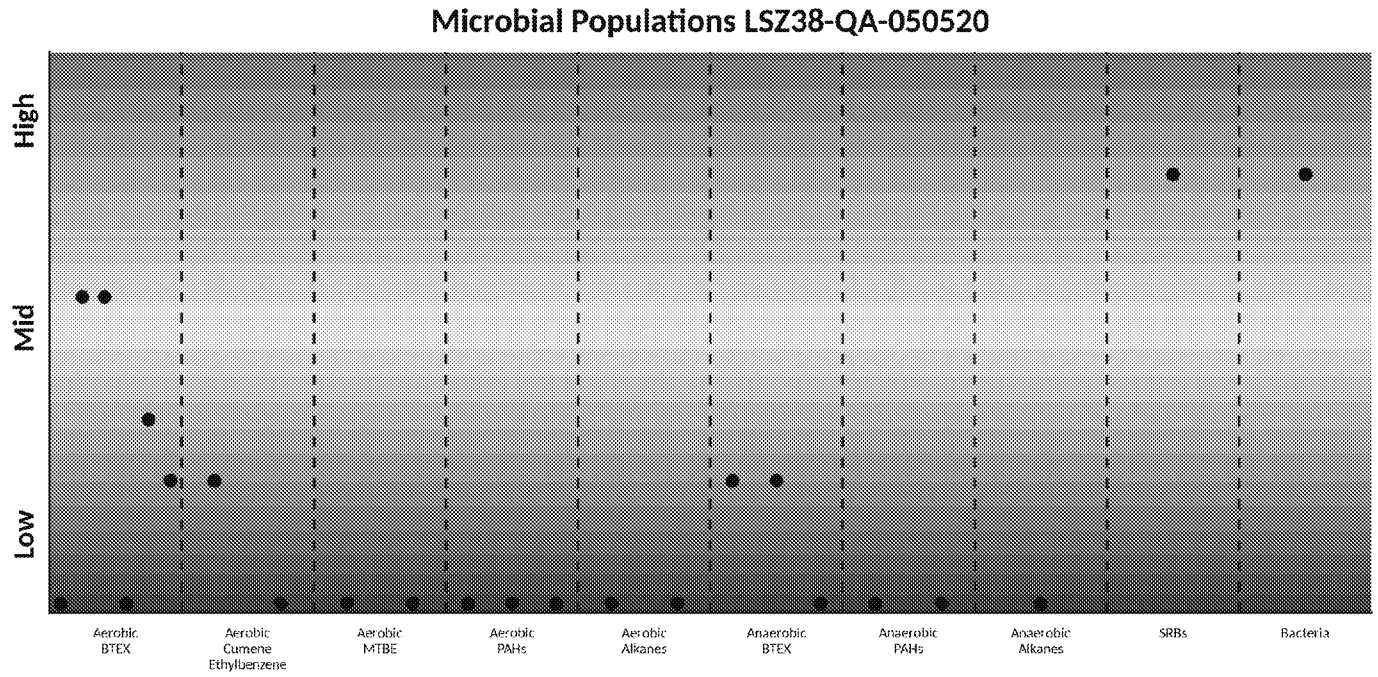

Figure 3: Microbial population summary to aid in evaluating potential pathways and biodegradation of specific contaminants.

Aerobic		Anaerobic	
BTEX	TOD, PHE, RDEG, RMO, TOL, EDO	BTEX	BCR, BSS, ABC
Cumene, Ethylbenzene	EDO, BPH4	Naphthalene/Methylnaphthalene	MNSSA, ANC
MTBE/TBA	PM1, TBA	Alkanes	assA
Naphthalene	NAH, NidA		
Phenanthrene	PHN		
Alkanes	ALK, ALMA		

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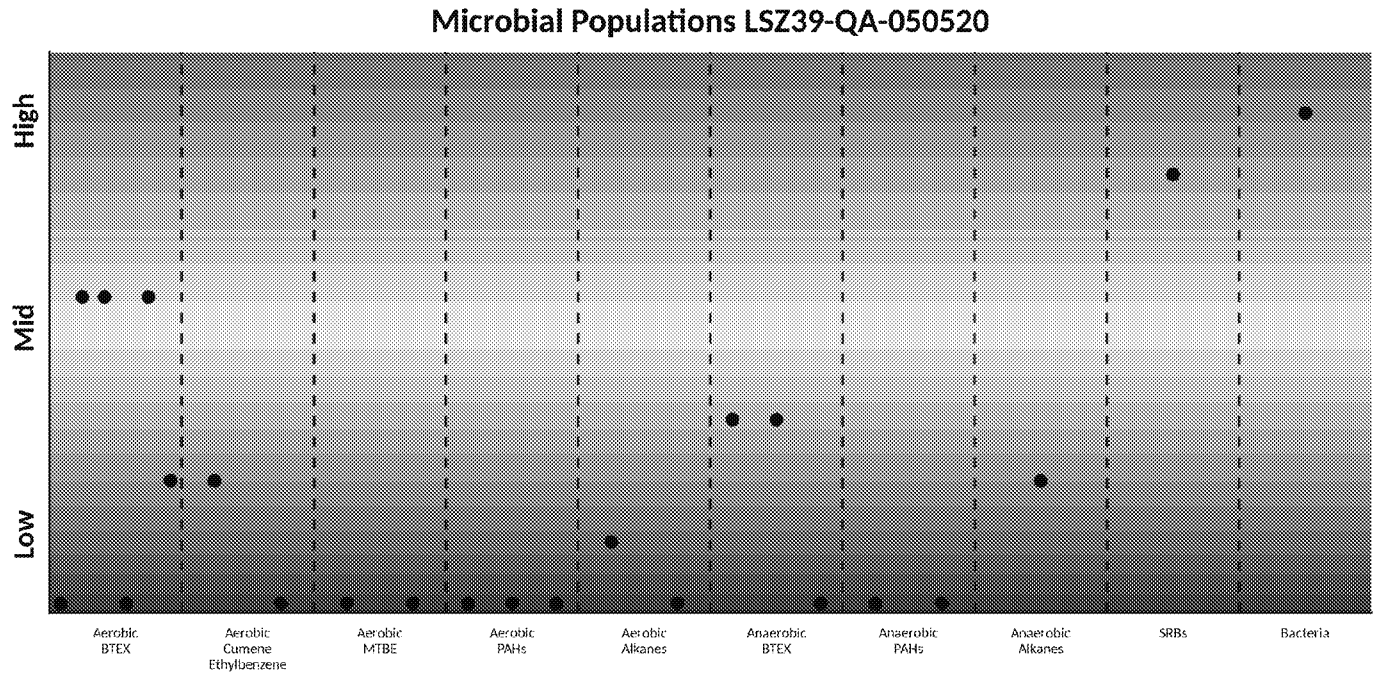

Figure 4: Microbial population summary to aid in evaluating potential pathways and biodegradation of specific contaminants.

Aerobic		Anaerobic	
BTEX	TOD, PHE, RDEG, RMO, TOL, EDO	BTEX	BCR, BSS, ABC
Cumene, Ethylbenzene	EDO, BPH4	Naphthalene/Methylnaphthalene	MNSSA, ANC
MTBE/TBA	PM1, TBA	Alkanes	assA
Naphthalene	NAH, NidA		
Phenanthrene	PHN		
Alkanes	ALK, ALMA		

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Table 2: Summary of the QuantArray®-Petro results for microorganisms responsible for aerobic biodegradation of BTEX and MTBE for samples UWBZ26-QA-050520, UWBZ27-QA-050520, LSZ38-QA-050520, and LSZ39-QA-050520.

Sample Name Sample Date	UWBZ26-QA-050520 05/05/2020	UWBZ27-QA-050520 05/05/2020	LSZ38-QA-050520 05/05/2020	LSZ39-QA-050520 05/05/2020
Aerobic BTEX and MTBE	cells/mL	cells/mL	cells/mL	cells/mL
Toluene/Benzene Dioxygenase (TOD)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Phenol Hydroxylase (PHE)	**2.36E+04**	**9.27E+04**	**4.22E+04**	**4.06E+04**
Toluene 2 Monooxygenase/Phenol Hydroxylase (RDEG)	**1.56E+04**	**6.66E+04**	**2.93E+04**	**2.37E+04**
Toluene Ring Hydroxylating Monooxygenases (RMO)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Xylene/Toluene Monooxygenase (TOL)	**1.68E+02**	**9.04E+02**	**2.52E+02**	**1.67E+03**
Ethylbenzene/Isopropylbenzene Dioxygenase (EDO)	**2.40E+00 (J)**	**9.40E+00**	**8.80E+01**	**7.17E+01**
Biphenyl/Isopropylbenzene Dioxygenase (BPH4)	<2.00E+01	**2.29E+01**	<2.27E+01	<2.27E+01
Methylibium petroleiphilum PM1 (PM1)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
TBA Monooxygenase (TBA)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01

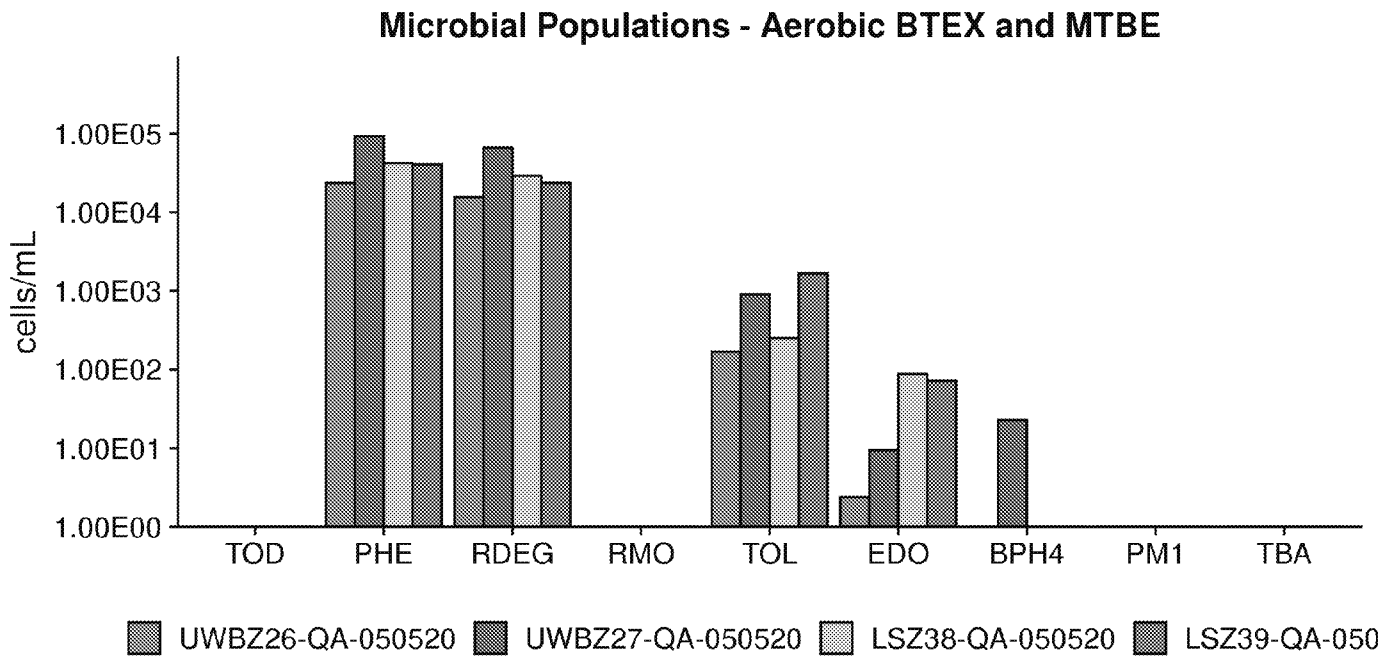

Figure 5: Comparison - microbial populations involved in aerobic biodegradation of BTEX and MTBE.

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Table 3: Summary of the QuantArray®-Petro results for microorganisms responsible for aerobic biodegradation of PAHs and alkanes for samples UWBZ26-QA-050520, UWBZ27-QA-050520, LSZ38-QA-050520, and LSZ39-QA-050520.

Sample Name Sample Date	UWBZ26-QA-050520 05/05/2020	UWBZ27-QA-050520 05/05/2020	LSZ38-QA-050520 05/05/2020	LSZ39-QA-050520 05/05/2020
Aerobic PAHs and Alkanes	cells/mL	cells/mL	cells/mL	cells/mL
Naphthalene Dioxygenase (NAH)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Naphthalene-inducible Dioxygenase (NidA)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Phenanthrene Dioxygenase (PHN)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Alkane Monooxygenase (ALK)	**7.00E+00 (J)**	<4.60E+00	<2.27E+01	**1.89E+01 (J)**
Alkane Monooxygenase (ALMA)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01

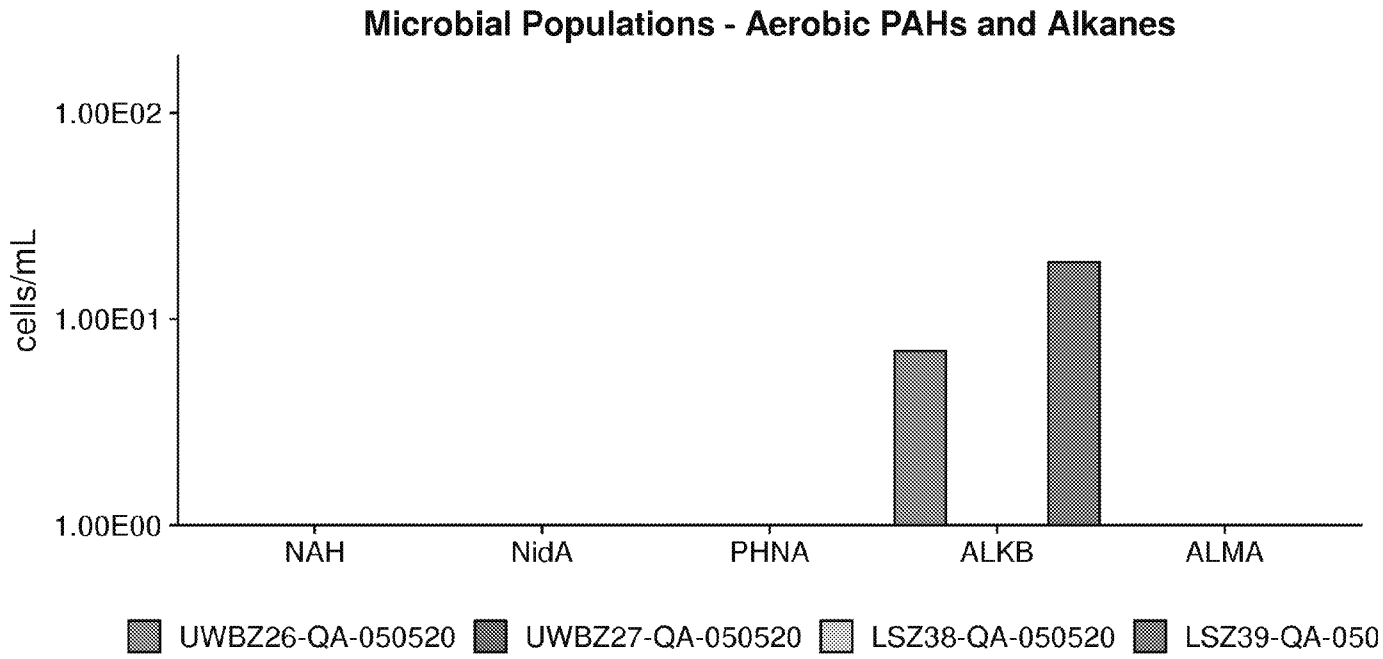

Figure 6: Comparison - microbial populations involved in aerobic biodegradation of PAHs and alkanes.

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Table 4: Summary of the QuantArray®-Petro results for microorganisms responsible for anaerobic biodegradation of BTEX, PAHs and alkanes for samples UWBZ26-QA-050520, UWBZ27-QA-050520, LSZ38-QA-050520, and LSZ39-QA-050520.

Sample Name Sample Date	UWBZ26-QA-050520 05/05/2020	UWBZ27-QA-050520 05/05/2020	LSZ38-QA-050520 05/05/2020	LSZ39-QA-050520 05/05/2020
Anaerobic BTEX	cells/mL	cells/mL	cells/mL	cells/mL
Benzoyl Coenzyme A Reductase (BCR)	**9.45E+02**	**3.19E+02**	**3.62E+01**	**9.49E+02**
Benzylsuccinate Synthase (BSS)	**1.04E+04**	**9.65E+02**	**8.50E+02**	**9.00E+03**
Benzene Carboxylase (ABC)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Anaerobic PAHs and Alkanes				
Naphthylmethylsuccinate Synthase (MNSSA)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Naphthalene Carboxylase (ANC)	<2.00E+01	<4.60E+00	<2.27E+01	<2.27E+01
Alkylsuccinate Synthase (ASS)	**1.20E+02**	**6.10E+00**	<2.27E+01	**1.14E+01 (J)**

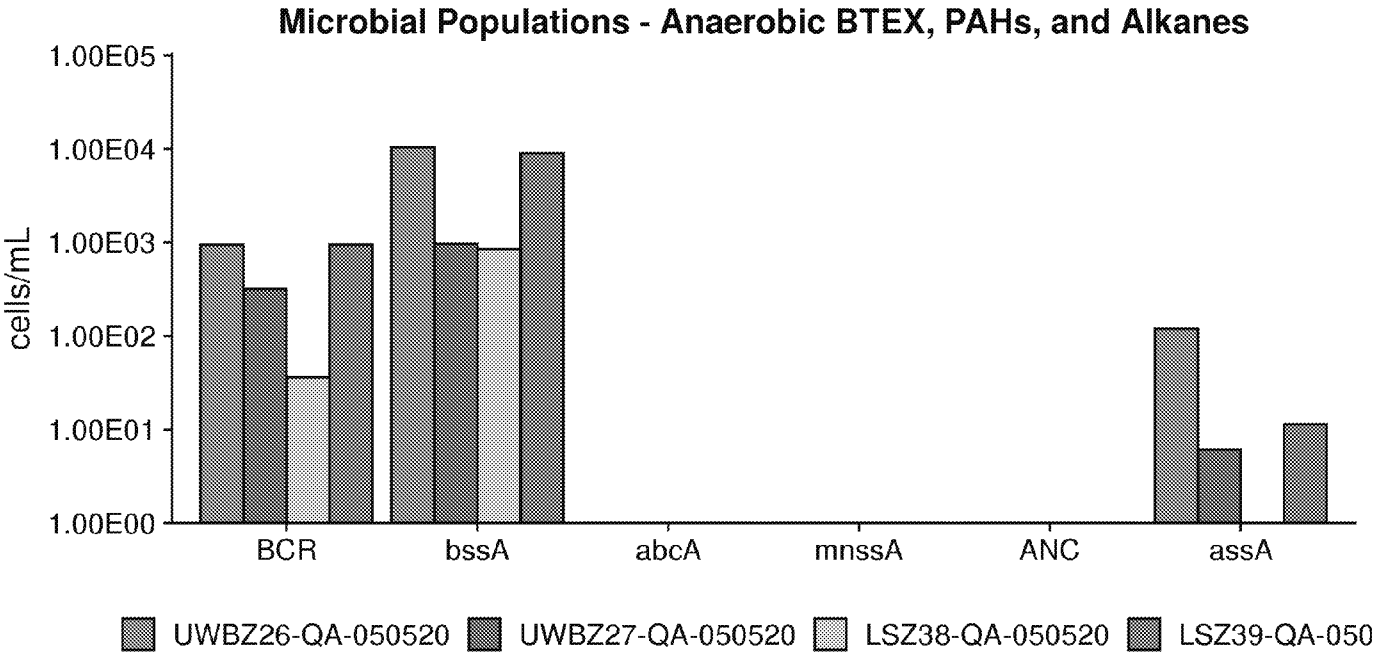

Figure 7: Comparison - microbial populations involved in anaerobic biodegradation of BTEX, PAHs and alkanes.

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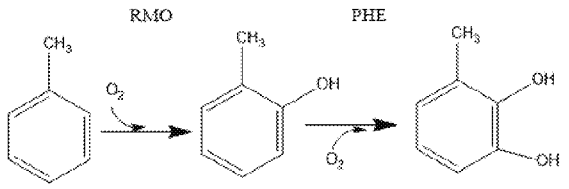
Interpretation

The overall purpose of the QuantArray®-Petro is to give site managers the ability to simultaneously yet economically evaluate the potential for biodegradation of a spectrum of contaminants found in petroleum products through a multitude of aerobic and anaerobic pathways to give a much more clear and comprehensive view of contaminant biodegradation. The following discussion describes interpretation of results in general terms and is meant to serve as a guide.

Aerobic Biodegradation - Benzene Toluene, Ethylbenzene, and Xylenes (BTEX): At sites impacted by petroleum products, aromatic hydrocarbons including BTEX are often contaminants of concern. Aerobic biodegradation of aromatic hydrocarbons has been intensively studied and multiple catabolic pathways have been well characterized. The substrate specificity of each pathway (range of compounds biodegraded via each pathway) is largely determined by the specificity of the initial oxygenase enzyme. The QuantArray®-Petro includes a suite of assays targeting the initial oxygenase genes of the known pathways for aerobic BTEX biodegradation.

Toluene/Benzene Dioxygenase (TOD): Toluene/benzene dioxygenase (TOD) incorporates both atoms of molecular oxygen into the aromatic ring. Although commonly called toluene dioxygenase, the substrate specificity of this enzyme is relaxed, allowing growth on toluene and benzene along with co-oxidation of a variety of compounds including ethylbenzene, *o*-xylene, *m*-xylene, and trichloroethene (TCE) when expressed.

Toluene/Benzene Monooxygenases (RMO/RDEG) and Phenol Hydroxylases (PHE): The next three known pathways for aerobic biodegradation of toluene (as well as benzene and xylenes) involve two steps: (1) an initial oxidation mediated by a toluene monooxygenase and (2) a second oxidation step catalyzed by a phenol hydroxylase. In these pathways, the toluene monooxygenases have been referred to as "ring hydroxylating monooxygenases" because they initiate biodegradation of toluene by incorporating oxygen directly into the aromatic ring rather than at a methyl group. The ring hydroxylating monooxygenases (RMOs) can be further described as toluene-2-monooxygenases, toluene-3-monooxygenases, or toluene-4-monooxygenases based upon where they attack the aromatic ring.



In General, phenol hydroxylases (PHE) catalyze the continued oxidation of phenols produced by RMOs. However, the difference between toluene monooxygenases (RMOs) and phenol hydroxylases (PHEs) is not absolute in terms of substrate specificity and catabolic function. For example, the TbmD toluene/benzene-2-monooxygenase [1] may be responsible for both the initial and second oxidation step [2].

The RMO, RDEG, and PHE assays target groups of genes encoding enzymes which perform the critical first and/or second steps in the aerobic biodegradation of BTEX compounds. In general terms, the RMO assay quantifies families of toluene-3-monooxygenase and toluene-4-monooxygenase genes. The RDEG assay is used to quantify groups of toluene-2-monooxygenase and phenol hydroxylase genes. Similarly, the PHE assay targets phenol hydroxylase genes and several benzene monooxygenase genes which catalyze both oxidation steps.

Toluene/Xylene Monooxygenase (TOL): The final known pathway for aerobic toluene biodegradation involves initial monooxygenase attack at the methyl group by a toluene/xylene monooxygenase.

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Ethylbenzene Dioxygenase (EDO): Similar to TOD, this group of aromatic oxygenases exhibits relatively broad specificity and is responsible for aerobic biodegradation of alkylbenzenes including ethylbenzene and isopropylbenzene or cumene [3].

Biphenyl Dioxygenase (BPH4): In environmental restoration, biphenyl dioxygenases are best known for cometabolism of polychlorinated biphenyls (PCBs). However, this subfamily includes benzene [4] and isopropylbenzene [5] dioxygenases from *Rhodococcus* spp.

Aerobic Biodegradation - MTBE and TBA: With increased use in the 1990s, the fuel oxygenate methyl *tert*-butyl ether (MTBE) has become one of the most commonly detected groundwater contaminants at gasoline contaminated sites. Pure cultures capable of utilizing MTBE as a growth supporting substrate have been isolated [6] and aerobic biodegradation of MTBE and the intermediate *tert*-butyl alcohol (TBA) has been reasonably well characterized. The QuantArray®-Petro includes quantification of two gene targets to assess the potential for aerobic biodegradation of MTBE and TBA.

Methylibium petroleiphilum PM1 (PM1): One of the few organisms isolated to date which is capable of utilizing MTBE and TBA as growth supporting substrates [6].

TBA Monooxygenase (TBA): Targets the TBA monooxygenase gene responsible for oxidation of TBA by *Methylibium petroleiphilum* PM1 [7].

Aerobic Biodegradation - Naphthalene and Other PAHs:

Naphthalene Dioxygenase (NAH): Naphthalene dioxygenase incorporates both atoms of molecular oxygen into naphthalene to initiate aerobic metabolism of the compound. However, the broad substrate specificity of naphthalene dioxygenase has been widely noted. When expressed, naphthalene dioxygenase is capable of catalyzing the oxidation of larger PAHs like anthracene, phenanthrene, acenaphthylene, fluorene, and acenaphthene. For a more comprehensive list of reactions mediated by naphthalene dioxygenases, see the University of Minnesota Biocatalysis/Biodegradation Database. (http://eawag-bbd.ethz.ch/naph/ndo.html, [8]).

Phenanthrene Dioxygenases (PHN): The PHN assays quantify phenanthrene/naphthalene dioxygenase genes from a diverse collection of microorganisms including *Pseudomonas*, *Burkholderia*, *Sphingomonas*, and *Acidovorax* spp. As with other naphthalene dioxygenases, substrate specificity is relatively broad and phenanthrene dioxygenases have been implicated in the biodegradation of naphthalene, phenanthrene, and anthracene and the co-oxidation of larger PAHs. Moreover, at least one research group has suggested that the PHN group of phenanthrene/naphthalene dioxygenases may be more environmentally relevant than the classical *nah*-like naphthalene dioxygenase [9].

Aerobic Biodegradation - *n*-alkanes: The *n*-alkanes are a substantial portion of petroleum products and are a component of TPH concentrations. The QuantArray®-Petro also includes quantification of alkane monooxygenase genes (ALK) which allow a wide range of *Proteobacteria* and *Actinomycetals* to grow on *n*-alkanes with carbon lengths from C_5 to C_{16} [10]. The QuantArray®-Petro also includes a second type of alkane hydroxylase (almA) which catalyzes the aerobic biodegradation of longer chain alkanes (C_{20}-C_{32}) by some *Alcanivorax* spp. considered dominant in marine systems [11].

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Anaerobic Biodegradation - Benzene, Toluene, Ethylbenzene, and Xylenes (BTEX): BTEX compounds are also susceptible to biodegradation under anoxic and anaerobic conditions although biodegradation pathways for each compound are not as well characterized as aerobic pathways. The QuantArray®-Petro includes sets of assays targeting a number of upper and lower pathway functional genes involved in the anaerobic catabolism of BTEX compounds for better evaluation of anaerobic biodegradation at petroleum contaminated sites.

Benzylsuccinate Synthase (BSS): Of the BTEX compounds, toluene biodegradation under anaerobic conditions is the most extensively studied and best characterized. The first step in this pathway, mediated by benzylsuccinate synthase (*bss*A) is the addition of fumarate onto the toluene methyl group to form benzylsuccinate. While additional pathways are possible, some bacterial isolates capable of anaerobic biodegradation of ethylbenzene and xylenes follow the same metabolic approach where the first step is the addition of fumarate.

Anaerobic Benzene Carboxylase (ABC): Although additional pathways are possible, the only pathway for anaerobic biodegradation of benzene elucidated to date is initiated by a benzene carboxylase enzyme.

Benzoyl Coenzyme A Reductase (BCR): Benzoyl-CoA is the central intermediate in the anaerobic biodegradation of many aromatic hydrocarbons. Benzoyl-CoA Reductase (BCR) is the essential enzyme for reducing the benzene ring structure.

Anaerobic Biodegradation - PAHs: The anaerobic biodegradation of PAHs involves analogous mechanisms to those described for anaerobic biodegradation of BTEX compounds. For example, the anaerobic biodegradation of methyl-substituted PAHs like 2-methylnaphthalene is initiated by fumarate addition to the methyl group while the only characterized pathway for anaerobic naphthalene biodegradation is initiated by a carboxylase.

Naphthylmethylsuccinate Synthase (MNSSA): MNSSA is analogous to the benzylsuccinate synthase described above for anaerobic biodegradation of toluene. Naphthylmethylsuccinate synthase catalyzes the addition of fumarate onto the methyl group of 2-methylnaphthalene [12].

Anaerobic Naphthalene Carboxylase (ANC): To date, the only pathway that has been characterized for anaerobic biodegradation of naphthalene is initiated by a naphthalene carboxylase enzyme [13].

Anaerobic Biodegradation - *n*-alkanes: As mentioned previously, the *n*-alkanes are a substantial portion of petroleum products and should be considered particularly when site cleanup goals include TPH reduction. The addition of fumarate is a common mechanism for activating and initiating biodegradation of a variety of petroleum hydrocarbons under anaerobic conditions including *n*-alkanes. The QuantArray®-Petro includes quantification of alkyl succinate synthase genes (assA) which have been characterized in nitrate reducing and sulfate reducing isolates utilizing *n*-alkanes from C_6 to at least C_{18} [14].

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